American Journal of Hematology 56:79–85 (1997)

Autocrine Activation of the IL-3/GM-CSF/IL-5 Signaling Pathway in Leukemic Cells

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The AML14.3D10 human myeloid leukemic cell line expresses receptors for granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-5 (IL-5), but not IL-3. We have found that this cell line produces GM-CSF in amounts up to 113 pg/ml in culture supernatants. Deprivation of endogenous GM-CSF by addition of neutralizing anti-GM-CSF antibody strongly inhibits proliferation of the cells, suggesting a GM-CSF autocrine growth mechanism. To examine whether endogenously produced GM-CSF activates intracellular GM-CSF/IL-3/IL-5-related signal transduction pathways, we performed anti-phosphotyrosine immunoblotting of cell lysates of AML14.3D10 cells before and after deprivation of endogenous GM-CSF. We found constitutive tyrosine-phosphorylation of a number of proteins in AML14.3D10 that could not be detectably increased by the addition of exogenous GM-CSF, IL-3, or IL-5. However, GM-CSF-deprived cells demonstrated a marked increase in phosphorylation of proteins of identical molecular mass following addition of GM-CSF and IL-5, but not IL-3, consistent with the receptor expression of the cells and the known use of the same signaling pathways by the three cytokines. This suggests that AML14.3D10 cells use endogenously produced GM-CSF to activate signal transduction pathways, interfering with activation by exogenous cytokine until the endogenous stimulation is removed. We then assessed the activation of the β-subunit common to the GM-CSF/IL-3/IL-5 receptors (βc), JAK2 and p53/56 *lyn,* known to be involved in the common signaling pathways of the three cytokines. We found that phosphorylation of βc and JAK2 in response to GM-CSF and IL-5 could be markedly enhanced by depriving cells of endogenous GM-CSF. Constitutive hyperphosphorylation of *lyn* was found in AML14.3D10 cells, and no further activation of *lyn* in response to cytokine was demonstrable in GM-CSF-deprived cells, suggesting that *lyn* is activated in this cell line by a mechanism other than GM-CSF. These studies represent the first demonstration of autocrine activation of intracellular cytokine signaling pathways by malignant hematopoietic cells. Because the addition of anti-GM-CSF to cell cultures improved responsiveness of intracellular signal transducing molecules to exogenous GM-CSF and IL-5, it can be inferred that endogenously produced GM-CSF exerts its effects by secretion and binding to surface GM-CSF receptors, although an intracellular component to signaling cannot be excluded. These observations provide further information regarding an autocrine contribution to leukemic cell growth, and establish a new model for study of these events. Am. J. Hematol. 56:79–85, 1997.

Key words: GM-CSF; signal transduction; autocrine growth

INTRODUCTION

The mechanisms by which leukemic cells maintain a transformed phenotype are poorly understood. A number of reports have demonstrated that leukemic cell lines or fresh leukemic blasts may produce hematopoietic growth factors that are known to augment leukemic cell growth

Contract grant sponsor: Department of Veterans Affairs Merit Review.

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Received 16 August 1996; Accepted 23 May 1997.

in vitro. This has led to speculation that autocrine pathways may be important in leukemic clonal expansion. However, the biologic relevance of these observations remains uncertain.

We have established a human myeloid leukemic cell line, AML14.3D10 [1], which expresses receptors for the hematopoietic growth factors GM-CSF and IL-5. This cell line also produces GM-CSF in amounts that could be expected to have significant biologic activity. These findings led us to examine whether AML14.3D10 cells could use endogenously produced GM-CSF to activate GM-CSF-related signal transduction pathways.

MATERIALS AND METHODS

The establishment and characterization of the AML14.3D10 cell line was previously described [1]. A monoclonal neutralizing anti-GM-CSF antibody was obtained from Pharmingen, San Diego, CA. Monoclonal neutralizing anti-IL-5 antibody was obtained from R&D Systems, Inc., Minneapolis, MN. The monoclonal anti-phosphotyrosine antibody 4G10 was obtained from UBI, Saranac, NY. A polyclonal antiserum reactive to the βc subunit shared by the GM-CSF, IL-3, and IL-5 receptors was obtained from UBI. Monoclonal antibodies reactive with JAK2 and the *src*-related kinase p53/65 *lyn* were obtained from UBI and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, respectively.

Production of GM-CSF by AML14.3D10 Cells

We have previously shown that AML14.3D10 cells produce mRNA for GM-CSF, but not IL-3, IL-5, or G-CSF [1]. The presence of GM-CSF in supernatants of AML14.3D10 grown to a density of 8×10^5/ml was assessed by ELISA, using a commercially available kit obtained from Endogen, Inc., Cambridge, MA, following the manufacturer's instructions.

Bioactivity of AML14.3D10-Derived GM-CSF

The cytokine-dependent TF-1 cell line [2] was incubated in a 96-well plate at a concentration of 5×10^3/well in either complete medium (RPMI containing 8% fetal calf serum, 2 mM glutamine, and 5×10^{-5} M β-mercaptoethanol) alone, complete medium containing 20 pg recombinant human GM-CSF, or 10% conditioned medium from AML14.3D10 cells grown to a density of 5×10^5/ml. Neutralizing anti-GM-CSF (80 ng/ml) or anti-IL-5 (80 ng/ml) was added to AML14.3D10-conditioned medium given to additional groups. All treatments were performed in replicates of six. Following a 48-hr incubation, wells were pulsed with 0.5 μCi [^{3}H]-thymidine and cultured for an additional 6 hr before harvesting onto glass fiber filters and scintillation counting. Results indicative of relative DNA synthesis are reported as mean counts per minute.

Effect of GM-CSF Deprivation on AML14.3D10 Cells

AML14.3D10 cells were cultured at a concentration of 1×10^4/well in a 96-well plate in either complete medium alone or in complete medium containing 80 ng/ml neutralizing anti-GM-CSF or 80 ng/ml anti-IL-5 for 12 hr before labeling with [^{3}H]-thymidine and assessment of incorporation into DNA by scintillation counting as described above. In another experiment, cells were cultured at 2×10^5/ml in 25 cm^2 tissue culture flasks alone or with the addition of 80 ng/ml anti-GM-CSF or anti-IL-5. Cells were counted and assessed for viability by trypan blue exclusion at 24 and 48 hr.

Effect of GM-CSF Deprivation on GM-CSF and IL-5 Signal Transduction in AML14.3D10 Cells

The receptors for GM-CSF, IL-3, and IL-5 are heterodimers consisting of an α-subunit unique for each cytokine and a shared β-subunit (βc) which is critical for signal transduction [3]. Stimulation of receptor-bearing cells with any of the three cytokines results in the tyrosine phosphorylation of a common set of cellular proteins [4–9] presumably because of the sharing of the signal-transducing βc by the three receptors. We have determined that AML14.3D10 cells express mRNA for βc, and for the α-subunits of GM-CSF and IL-5, but not IL-3. Cell surface binding of fluorochrome-conjugated GM-CSF and IL-5 has been demonstrated by flow cytometric study (data not shown).

Tryosine phosphorylation of intracellular proteins of AML14.3D10 cells incubated for varying periods of time with receptor saturating concentrations of GM-CSF, IL-3, and IL-5, was assessed by Western blotting of cell lysates with an anti-phosphotyrosine antibody (4G10). Briefly, 8×10^5 cells were incubated at 37°C for 1, 5, and 10 min with 10 ng/ml GM-CSF, 10 ng/ml IL-3, or 20 ng/ml IL-5 before being placed in ice cold lysis buffer containing protease and phosphatase inhibitors (20 mM Tris, pH 8.0, containing 137 mM NaCl, 10% glycerol, 1% NP40, 10 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 2 mM sodium orthovanadate, 0.15 U/ml aprotinin, and 10 μg/ml leupeptin) for 20 min. Cell lysates were boiled in SDS, subjected to electrophoresis through 2–8% polyacrylamide gels, and transferred to nylon membranes. Membranes were blocked with 1% BSA in 20 mM Tris-base, pH 7.6, 137 mM NaCl, 0.1% Tween-20 (TBST) for 30 min before 90-min incubation at room temperature with 1:2,000 4G10 antibody in TBST. Bound antibody was detected by chemiluminescence, using a kit obtained from Amersham Life Science Inc., Arlington Heights, IL, following the manufacturer's instructions. Duplicate experiments were performed, using AML14.3D10 cells that had been incubated for 48 hr in 80 ng/ml neutralizing anti-GM-CSF, to deplete endogenously produced GM-CSF.

The receptors for GM-CSF, IL-3, and IL-5 possess no intrinsic kinase activity [3]. Signaling by these receptors following ligand binding is thought to occur via recruitment of kinases with initial tyrosine phosphorylation of βc and subsequent phosphorylation of a number of other kinases. We examined AML14.3D10 cells for the involvement of kinases previously reported to be activated by the three cytokines by immunoprecipitating cytokine-stimulated cell lysates prepared as described above with antibodies to βc, JAK2, and the *src*-related kinase p53/56 *lyn* in the presence of protein A agarose beads. Immunoprecipitates were subjected to polyacrylamide gel electrophoresis and Western blotted with antiphosphotyrosine as described above. Duplicate experiments were performed using AML14.3D10 cells that had been incubated for 48 hr with neutralizing anti-GM-CSF.

RESULTS

Production of Bioactive GM-CSF by AML14.3D10 Cells

ELISA demonstrated that AML14.3D10 cells grown to a concentration of 8×10^5/ml secrete immunoreactive GM-CSF to a concentration of 113 pg/ml, a range that would be expected to produce significant biologic effects given the reported dissociation constant of the GM-CSF receptor [10]. Bioactivity of AML14.3D10-derived GM-CSF was confirmed by demonstration of augmented proliferation of the GM-CSF-responsive TF-1 cell line, with specific inhibition of activity by neutralizing anti-GM-CSF, but not anti-IL-5 (Fig. 1).

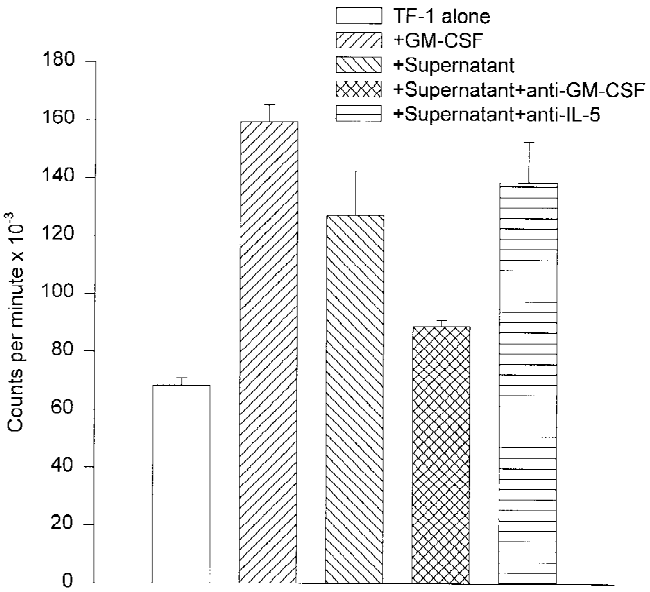

Fig. 1. Bioactivity of GM-CSF produced by AML14.3D10 cells. TF-1 cell proliferation is stimulated by recombinant human GM-CSF and AML14.3D10 culture supernatant. Stimulation is reduced by preincubation of supernatant with anti-GM-CSF, but not anti-IL-5. Results are graphed as mean counts per minute of 6 replicates ± S.D.

Effect of GM-CSF Deprivation on AML14.3D10 Cells

Incubation of AML14.3D10 cells with neutralizing anti-GM-CSF significantly and specifically impaired proliferation, as assessed by thymidine incorporation (Fig. 2) and by cell counting at 24 and 48 hr, which showed a 25% reduction in the number of cells in the anti-GM-CSF supplemented flask at both time points compared to the unsupplemented flask. There was no difference in cell count between the unsupplemented flask and the anti-IL-5 supplemented flask. Viability was 98–99% in all flasks at both time points.

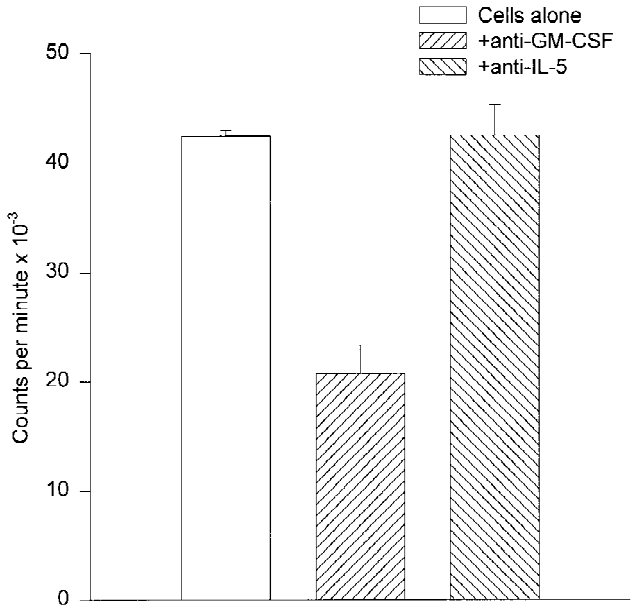

Fig. 2. Inhibition of proliferation of AML14.3D10 cells by anti-GM-CSF, but not anti-IL-5. Results are graphed as mean counts per minute of 6 replicates ± S.D.

Effect of GM-CSF Deprivation on GM-CSF and IL-5 Signal Transduction

Antiphosphotyrosine Western blotting demonstrated that AML14.3D10 cells constitutively contain a number of detectable tyrosine phosphorylated species. Addition of exogenous GM-CSF, IL-3, or IL-5 did not cause a significant increase in phosphorylation (Fig. 3, top). Remarkably, following 48-hr incubation with anti-GM-CSF, exposure of the cells to exogenous GM-CSF or IL-5, but not IL-3, resulted in a rapid, marked increase in tyrosine phosphorylation of several species (Fig. 3, bottom). Two of the more prominent were seen at approximately 130 and 140 kD, which is consistent with the reported molecular mass of phosphorylated JAK2 [11] and βc [12].

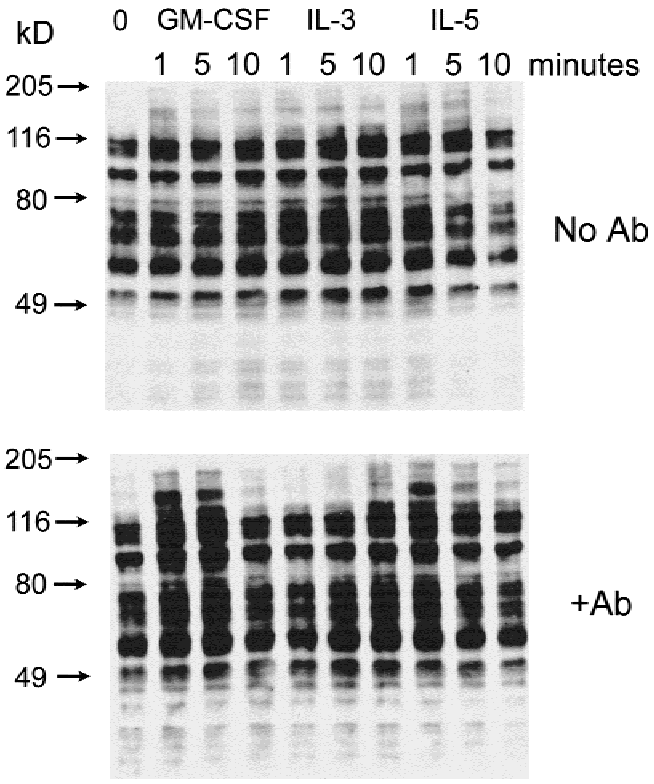

Fig. 3. Anti-phosphotyrosine Western blots of cell lysates of AML14.3D10 cells incubated for varying periods of time in receptor-saturating concentrations of GM-CSF, IL-3, and IL-5. Top: AML14.3D10 cells. Bottom: Cells that had been pre-incubated for 48 hr in 80 ng/ml neutralizing anti-GM-CSF antibody.

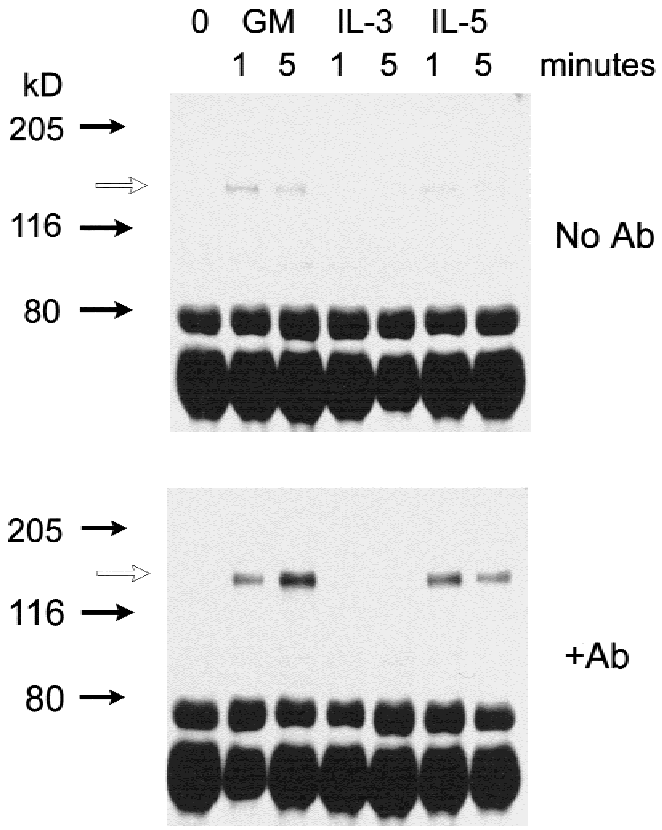

Fig. 4. Anti-phosphotyrosine Western blots of lysates of AML14.3D10 cells that had been immunoprecipitated with anti-βc following stimulation for varying times with cytokine. Top: AML14.3D10 cells. Bottom: Cells that had been preincubated for 48 hr in 80 ng/ml neutralizing anti-GM-CSF antibody. The open arrows show the position of βc.

Although some increase in phosphorylation of βc was detectable in βc immunoprecipitates of AML14.3D10 cells following stimulation with GM-CSF and IL-5, phosphorylation in response to the cytokines was markedly enhanced in cells preincubated for 48 hr with anti-GM-CSF (Fig. 4). Little or no phosphorylation of JAK2 could be detected in JAK2 immunoprecipitates of antibody untreated cells stimulated with cytokines, but antibody-treated cells responded to GM-CSF and IL-5 by increasing phosphorylation of JAK2 (Fig. 5). Interestingly, phosphorylation of p53/56 *lyn* appeared to be constitutive in AML14.3D10 cells, and was not significantly enhanced by cytokine stimulation regardless of whether cells had been pre-incubated with anti-GM-CSF (Fig. 6).

DISCUSSION

To our knowledge, these studies represent the first demonstration of autocrine activation of intracellular cytokine signaling pathways by malignant hematopoietic cells. These observations provide further evidence in support of an autocrine contribution to leukemic cell growth, and establish a new model for study of these events.

A number of previous in vitro studies have demonstrated that a substantial proportion of fresh leukemic blasts or leukemic cell lines may produce hematopoietic growth factors, bear receptors for the produced factors, and be growth inhibited by neutralization of the produced factors [13–19]. Our studies extend these observations by demonstrating that AML14.3D10 cells use endogenously produced GM-CSF to activate intracellular GM-CSF-related signal transduction pathways. The minimal increase in tyrosine phosphorylation of intracellular substrates in response to GM-CSF and IL-5 until cells are pre-treated with neutralizing anti-GM-CSF suggests that endogenously produced GM-CSF has activated signaling pathways, interfering with an effect of additional exogenous cytokine. Because GM-CSF, IL-3, and IL-5 are known to activate identical signaling molecules [3], the reduced response to IL-5 until cells are pre-treated with anti-GM-CSF is consistent with this interpretation. Lack of response to IL-3 either before or after endogenous

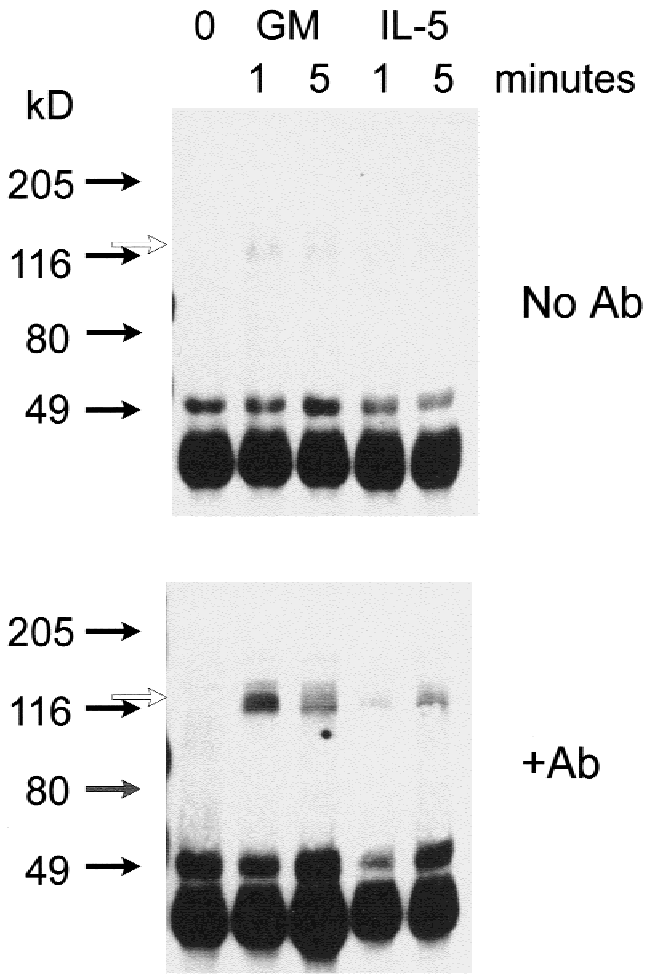

Fig. 5. Anti-phosphotyrosine Western blots of lysates of AML14.3D10 cells that had been immunoprecipitated with anti-JAK2 following stimulation for varying times with cytokine. Top: AML14.3D10 cells. Bottom: Cells that had been preincubated for 48 hr in 80 ng/ml neutralizing anti-GM-CSF antibody. The open arrows show the position of JAK2. The additional heavier band seen at approximately 140 kD is likely co-precipitated βc, as has been described by others [21].

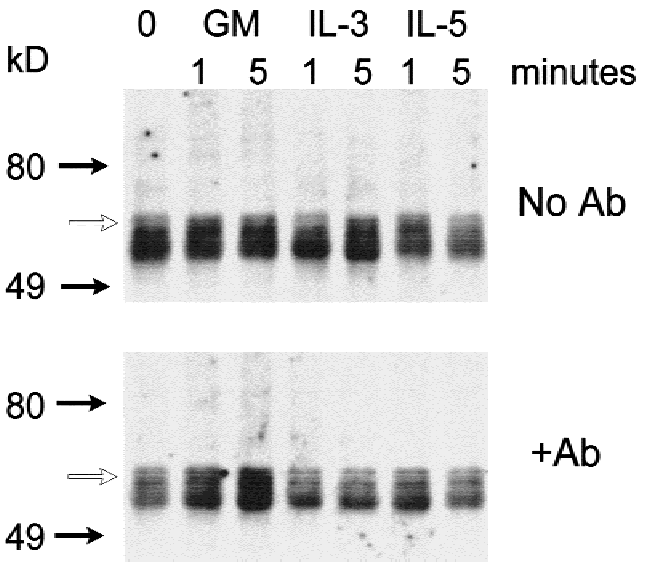

Fig. 6. Anti-phosphotyrosine Western blots of lysates of AML14.3D10 cells that had been immunoprecipitated with anti-*lyn* following stimulation for varying times with cytokine. Top: AML14.3D10 cells. Bottom: Cells that had been preincubated for 48 hr in 80 ng/ml neutralizing anti-GM-CSF antibody. The open arrows show the position of the *lyn* doublet.

GM-CSF depletion is presumably related to the lack of expression of the IL-3 receptor α-subunit by AML14.3D10 cells, and, thus, the lack of a functional IL-3 receptor.

Previous studies have identified several of the kinases involved in GM-CSF, IL-3, and IL-5 signaling. Although the receptors for these cytokines have no apparent intrinsic kinase activity [3], the β-subunit shared by the three receptors rapidly becomes tyrosine-phosphorylated following ligand binding [7,12], presumably due to recruitment of cytoplasmic kinases. The JAK2 kinase has also been shown to become activated by tyrosine phosphorylation following GM-CSF/IL-3/IL-5 binding [11,20], and is thought to become associated with βc [21]. Our studies demonstrate that depletion of endogenously produced GM-CSF from AML14.3D10 cells permits augmented tyrosine phosphorylation of βc and JAK2 by exogenous GM-CSF and IL-5, again consistent with the idea that activation of these substrates by endogenously produced GM-CSF may limit their availability for exogenous activation. The apparent lack of heavy constitutive phosphorylation of βc and JAK2 in AML14.3D10 cells may be the result of activation of phosphatases [22,23] in response to the persistent endogenous kinase activity.

The src-related kinase p53/56 *lyn* has been previously shown to be involved in the GM-CSF/IL-3/IL-5 signaling pathway [8,9]. Our studies suggest that *lyn* is constitutively activated in AML14.3D10 cells, and that depletion of endogenous GM-CSF does not result in enhanced activation of *lyn* by exogenous GM-CSF or IL-5. This suggests that *lyn* may be activated in these cells by a stimulus other than GM-CSF. This event may be important to the transformed phenotype of this cell line. It is clear from transfection studies and from transgenic models that autocrine use of hematopoietic cytokines alone is insufficient to produce a malignant phenotype [24]. Others have also found constitutive hyperphosphorylation of substrates [25] known to be part of cytokine signal transduction pathways [26,27] in cell lines that are cytokine-independent, suggesting that derangements resulting in such activation may be important in malignant transformation. The relative contributions of autocrine GM-CSF activity, *lyn* activation by alternative means, and possible

additional mechanisms to the malignant phenotype exhibited by AML14.3D10 cells remain to be established and should add to our knowledge of the mechanisms of leukemogenesis.

It has been unclear whether hematopoietic growth factors might participate in autocrine growth stimulation by totally intracellular mechanisms [28,29], or whether secretion of the factor and engagement of surface-expressed receptors is required [30,31]. Our observations of anti-GM-CSF inhibition of cell proliferation and concomitant increased availability of intracellular signal transducing molecules to activation by exogenously added GM-CSF or IL-5 support a mechanism in which secretion and utilization of surface receptors is operative, although an intracellular component to signaling cannot be excluded.

The transcriptional regulation of the GM-CSF gene is complex and may be largely negative, given the strong constitutive activity in many tissues of recently described upstream enhancer elements [32]. The AML14.3D10 cell line will be valuable for examining the molecular mechanisms leading to dysregulation of GM-CSF expression.

ACKNOWLEDGMENTS

This work was supported by the Department of Veterans Affairs Merit Review funding to Drs. Paul and Baumann.

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